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Breast cancer is frequently associated with gene amplification at the chromosome 11q13. Studies have demonstrated that cortactin (also EMS1), a filamentous actin (F-actin) associated protein and a substrate of protein tyrosine kinase Src, plays an important role in the amplification. However, the pathological role of cortactin in breast cancer is unknown. We have developed a form of cortactin mutant that is deficient in tyrosine phosphorylation and is no longer to be regulated by Src. The mutant and wild-type forms of cortactin and oncogenic Src were introduced into MDA-MB-231 cells, a highly invasive breast caner cell line. Overexpression of wild-type cortactin or oncogenic Src potentiate the motility of the cells, while overexpression of the mutant form impairs the cell migration as well as cell invasion into extracellular matrix. This study indicates that cortactin may play an important role in metastasis associated with breast cancer.

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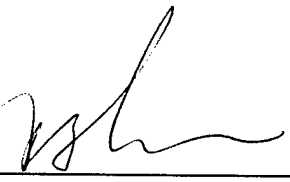

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INTRODUCTION

Breast cancer is frequently associated with gene amplification at the chromosome 11q13. Studies have demonstrated that cortactin (also EMS1), a filamentous actin (F-actin) associated protein and a substrate of Src, plays an important role in the amplification. However, the pathological role of cortactin in breast cancer is unknown. Our previous study has shown that cortactin is implicated in cell migration and cytoskeletal reorganization. We hypothesized that cortactin plays also a role in metastasis. To test this hypothesis, we have proposed the following tasks: (1) establish cortactin mutants that can alter the function of the endogenous cortactin and (2) analyze the effect of the mutant on the invasive ability of breast tumor cells in vitro and in vivo. We anticipate that establishment of the role of cortactin cell invasion will provide a novel approach to design drugs that will target specifically on a cytoskeletal protein and eventually to prevent metastasis, the major cause for the death associated with breast cancer.

BODY

Task 1.) Establish cortactin mutants that can alter the function of the endogenous cortactin

Cortactin is able to cross-link F-actin, and this cross-linking activity can be down-regulated by Src (1). Src phosphorylates three tyrosine residues of cortactin: Tyr421, Tyr466 and Tyr482. We recently constructed a form of myc-tagged cortactin mutant (myc-$Cort_{F421F466F482}$) that is deficient in tyrosine phosphorylation mediated by Src (2). As expected, we found that the mutant is no longer down-regulated by Src for the F-actin cross-linking (2). To test whether the mutant can function as a dominant negative mutant within cells, we transfected the mutant into an endothelial cell line ECV304. What we found was that the cells overexpressing the wild-type cortactin has acquired increased cell motility. In contrast, the cells overexpressing the mutant shows a reduced cell motility (2). Thus, the cortactin variant lacking of tyrosine phosphorylation indeed can function as a dominant negative mutant.

Taks 2.) Analyze the effect of the mutant on the invasive ability of breast tumor cells in vitro.

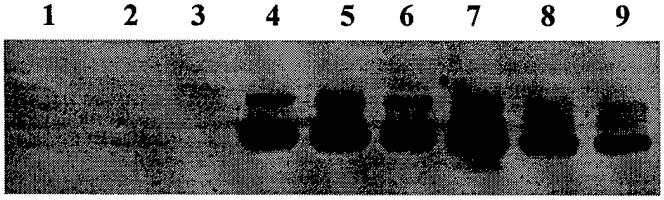

Figure 1. Expression of cortactin in MDA-MB-231 cells. MDA-MB-231 cells were transfected with myc-tagged cortactin constructs or the vector (pMEXneo) alone. Stable transfectants were selected in G418 containing medium. Stable transfectants were lysed and analyzed by immunoblotting using monoclonal antibody 9E10 against the myc epitope. Lanes 1-3, MDA/pmex-neo, clone 1, 2 and 3; lanes 4-6, MDA/myc-cortactin, clone 1, 2, and 3; lanes 7-9, MDA/myc-$cort_{F421F466F482}$, clone 1, 2 and 3.

We have transfected both wild-type and mutated forms of cortactin into MDA-MB-231, a highly invasive breast cell line. The expression levels of the cortactin forms were determined by immunoblotting analysis (Figure 1). The established stable MDA overexpressors were then analyzed for the ability to migrate into a wounded area introduced by a rubber policemen. As shown in Figure 2, the wild-type cortactin overexpressors have increased the motility by nearly two-folds compared to cells expressing the vector, and the mutant overexpressors have reduced motility by nearly 50%. Hence, tyrosine phosphorylation of cortactin plays an important role in tumor cell motility. Consistent with this is that cells expressing an oncogenic form of Src (v-Src) shows even higher motility than cells expressing wild-type cortactin (Figure 3).

The motility of cortactin expressors was also analyzed using Transwell (Costar), a modified Boyden Champber. As shown in Figure 3, the similar result as wound-healing assay was obtained. The mutant transfectant had motility about 33% less than control

cells, whereas wild-type cortactin transfectant showed motility about 50% higher than control cells.

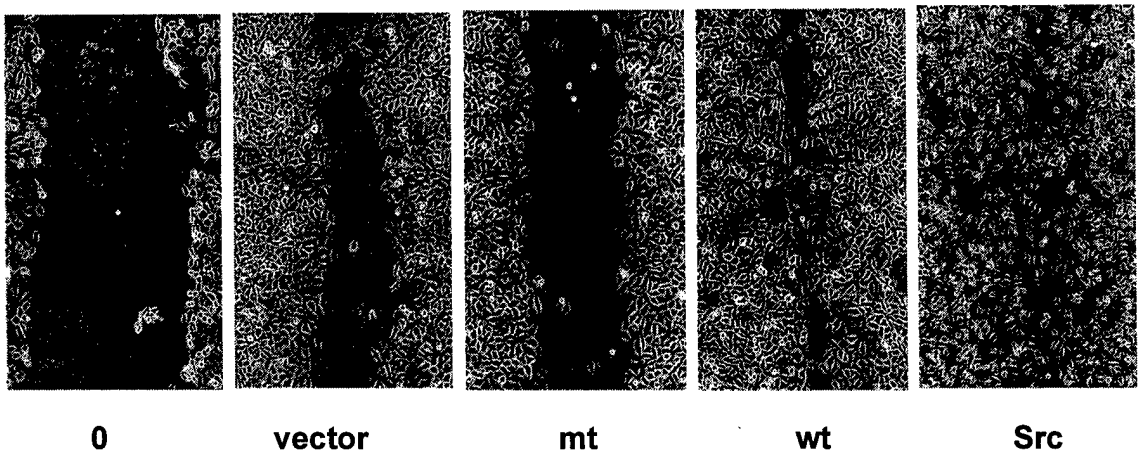

Figure 2. Cortactin mutant impairs the migration of MDA-MB-231 cells. Confluent MDA-MD-231 cells expressing cortactin variants or oncogenic Src were scraped with a plastic gel spacer (0.75 mm). After 20 h, the wounded areas were photographed.

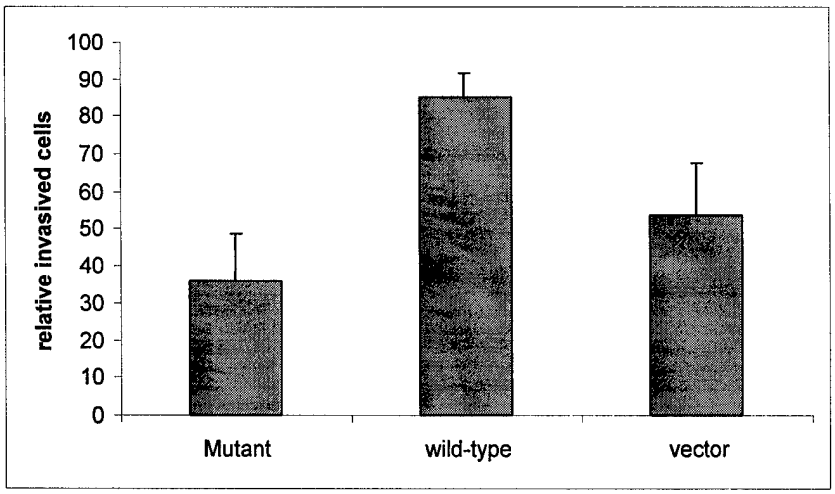

Figure 3. Analysis of MDA-MB-231 cells with Transwell chamber. Stable MDA-MB-231 transfectants migration was examined in transwell cell-culture chambers (Costar, Cambridge, MA). Polycarbonate membrane inserts with 8.0μm was coated on the lower side with fibronectin (50μg/ml) overnight. Sub-confluent MDA-MB-231 cells were trypsinized, washed one time with DMEM containing 0.2% soybean trypsin inhibitor and one time with DMEM containing 0.4% fetal bovine serum (FBS). The cells were resuspended in DMEM plus 0.4% FBS and plated on the upper chamber at a density of 50,000 cells per well. The lower compartment was also added with 0.6 ml DMEM plus 0.4% FBS. The cells were incubated at 37°C. After 5 hours, the membranes were washed one time with PBS and subsequently fixed with methanol for 10 min at 4°C. The cells were counterstained with hematoxylin. The upper side of insert was wiped with a cotton swab. The number of MDA-MB-231 cells that migrated to the lower side of the membranes was determined microscopically. The data shown represent average of six independent experiments.

We have also set up a collaboration with Dr. Susette Mueller in Georgetown University to examine the invasive ability of cortactin expressors. In this assay, the invasive ability of cells was measured by the degradation of gelatin (3). Cells expressing high levels of cortactin mutant caused much less degradation of extracellular substratum than cells expressing wild-type cortactin.

KEY RESEARCH ACCOMPLISHMENTS:

1. Established a form of cortactin that can function as dominant negative mutant
2. Demonstrate cortactin is implicated in the motility of breast tumor cells

REPORTABLE OUTCOMES

1. Manuscripts:
   - Src is required for cell migration and shape changes induced by fibroblast growth factor 1 (enclosed in Appendix)
   - The mechanism regulating the filamentous actin cross-linking activity of cortactin by Src (enclosed in Appendix)
2. Degrees obtained: nearly 50%
3. Development of cell lines:
   - MDA-MB-231 cells expressing wild-type and mutated forms of cortactin

CONCLUSIONS

We have developed a form of cortactin mutant that is deficient in tyrosine phosphorylation. Overexpression of the mutant in breast cancer cells, MDA-MB-231, can impair both cell migration and cell invasion as examined by in vitro assays. This result demonstrates that cortactin plays an important role in cell migration and cell invasion. This preliminary result encourages us to further test the effect of the cortactin proteins on metastasis in an animal model, which we plant to perform in the next fiscal year.

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3. Coopman, P.J., Do, M.T., Thompson, E.W., and Mueller, S.C. (1998) *Clin.Cancer Res.* **4** , 507-515

APPENDICES:

1. A reprint for the paper entitled "Src is required for cell migration and shape changes induced by fibroblast growth factor 1"

2. A copy of the manuscript entitled "The mechanism regulating the filamentous actin cross-linking activity of cortactin by Src"

Submitted to J. Biol. Chem.

The mechanism regulating the filamentous actin cross-linking activity of cortactin by Src

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Running Title: Down-regulation of the dimerization of cortactin by tyrosine phosphorylation

Abbreviations: β-gal, β-galactosidase; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; F-actin, filamentous actin; FGF, fibroblast growth factor; GST, glutathione S-transferase; NHS, N-hydroxysulfosuccinimide; ONPG, o-nitrophenyl β-D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; and SH3, Src homology 3.

This study was supported by National Institutes of Health Grant HL52753 and Department of the Army grant DAMD17-98-1-8278 (to X.Z.)

ABSTRACT

Protein tyrosine kinase Src regulates cytoskeletal reorganization by attenuating the function of cortactin, a filamentous actin cross-linking protein within the cell cortex. Cortactin normally forms a homo-dimer. A mutant where the Src-targeted tyrosine residues were replaced with aspartic acids acquires a similar property as phosphorylated cortactin and cross-links F-actin less efficiently than wild-type cortactin. This reduced F-actin cross-linking activity is correlated with the inability of the mutant to form a dimer. Furthermore, Src inhibits directly cortactin dimerization in vitro. Thus, cortactin dimerization is required for its apparent F-actin cross-linking activity, and disruption of its dimerization by tyrosine phosphorylation is the mechanism for Src-mediated attenuation of the F-actin cross-linking activity of cortactin.

INTRODUCTION

Cell migration and cell shape changes induced by extracellular stimuli require dynamic cytoskeletal reorganization involving actin polymerization, filamentous actin (F-actin) cross-linking and retrograde actin flow (1). Whereas recent study has pointed the role of Ras-related small GTPases in the regulation of stress fibers, lamellipodia and filopodia (2), the mechanism by which the cytoskeletal rearrangement occurs in response to extracellular signals is still elusive. In addition to small G proteins, Src-related protein tyrosine kinases are also implicated in the cytoskeletal reorganization. The preferred substrates of Src within cells are almost exclusively comprised of those molecules that associate with the actin cytoskeleton or focal adhesion sites. These include cortactin, $p190^{RhoGAP}$, $p120^{FAK}$ and $p130^{CAS}$ (3). Indeed, these cytoskeletal proteins are known to act as modulators in the formation of adhesion plaques and cell cortical structures. It is also established that these proteins are often subjected to phosphorylation at tyrosine residues upon stimulation by extracellular ligands. However, the significance of tyrosine phosphorylation of these molecules by Src in the cytoskeletal reorganization and the mechanism for tyrosine phosphorylated proteins to regulate the cytoskeleton are not understood.

Cortactin, which is widely expressed in most adherent cells (4), appears to be a major substrate of Src in vivo (5). Cortactin binds to F-actin directly and co-localizes with activated Src within podosome, a membrane-substratum interaction area (5;6). In addition, tyrosine phosphorylation of cortactin can be also regulated by growth factors including fibroblast growth factor 1 (FGF-1) (7), epidermal growth factor (EGF) (8) and platelet-derived growth factor (PDGF) (9). In normal quiescent cells, cortactin is primarily associated with a punctate-like structure in the cytoplasm (6;10). In response to growth factors such as PDGF and FGF-1, cortactin is phosphorylated at tyrosine residues and translocated into the leading edge of cells including lamellipodia and membrane ruffles (ref 11 and J. Liu, unpublished result). In a recent study, we showed that FGF-mediated cell migration requires a functional Src and correlates with the tyrosine phosphorylation of cortactin (12). In addition, overexpression of wide-type cortactin potentiates cell migration (9;10), whereas overexpression of a cortactin variant with mutation at the tyrosine residues targeted by Src impairs the cell migration (10). Furthermore, cortactin is impaired for tyrosine phosphorylation and is unable to associate with polarized lamellipodia within cells deficient in Src (12). These findings imply that tyrosine phosphorylation plays a regulatory role in the function of cortactin. In a previous study we demonstrated that cortactin is an F-actin cross-linking protein, which can be down regulated upon tyrosine phosphorylation by Src (13). However, the mechanism by which tyrosine phosphorylation attenuates the activity of cortactin is unknown.

The protein sequence of cortactin is featured by six copies of a unique 37-amino-acid repeat and a Src homology 3 (SH3) domain at the COOH terminus. Between the repeat and the SH3 domain there is an alpha-helical structure followed by a proline-rich sequence. Whereas cortactin contains total 27 tyrosine residues, only three of them (Tyr-421, Tyr-466 and Tyr-482, which are located between the proline-rich and the SH3 domain) can be efficiently phosphorylated by Src in vitro and in vivo (10). To be an F-actin cross-linker, the protein requires at least two independent F-actin binding sites (14). While it has been reported that the repeat domain of cortactin is responsible for F-actin binding, the mutant

bearing only three repeat units is not sufficient for F-actin binding (6), indicating that cortactin has only a single motif for F-actin binding. Because many actin cross-linkers such as spectrin and ABP-280 acquire two F-actin binding sites through dimerization (15;16), we thought that cortactin could form a dimer. In this report, we provide evidence that cortactin can form a homo-dimer in vitro. Replacing tyrosine residues targeted by Src with aspartic acids can impair the dimerization of cortactin. In addition, the mutant bearing transition from tyrosine to aspartic acid mimics tyrosine phosphorylated wild-type cortactin and acquires a reduced activity for F-actin cross-linking. Furthermore, Src can directly inhibit the dimerization of cortactin. This finding demonstrates a novel mechanism by which tyrosine phosphorylation mediated by Src regulates the cytoskeleton by virtue of disruption of the cortactin dimerization.

EXPERIMENTAL PROCEDURES:

Site-directed mutagenesis and Plasmids preparation

Mutations that substitute Tyr-421 and Tyr-466 with aspartic acids were generated by polymerase chain reaction (PCR) using ExSite site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instruction. Two pairs of deoxyoligonucleotides were used as primers in PCR: GACGAGGATGCAGCTCCGTT/GATGGGGCTGGAGGGTGGTC and GACTACAGAGGCTCCTGGC/TCGTCCACGGGCTCTGATG. The mutations were confirmed by DNA sequencing. The PCR-generated fragments were used to replace the *Sac*1 and *EcoR*1 digested fragment of a GST-cortactin plasmid as described previously (13). The resulting mutant protein is designated as $Cort_{D421D466}$. The Tyr-482 and Tyr-485 of $Cort_{D421D466}$ were further substituted with aspartic acids by using QuickChange site-directed mutageneis kit (Stratagene, La Jolla, CA). The primers used in PCR were GAGGATGACACCGACGATGGGGATGAGAGT/GTCACTCTCATCCCCATCGTCGGTGTCATC. The mutations were confirmed by DNA sequencing. The resulting mutant protein is designated as $Cort_{D421D466D482D485}$.

The plasmids for the two-hybrid yeast analysis were prepared as follows. The DNA fragments encoding wild-type cortactin and $Cort_{D421D466}$ were amplified by PCR using pfu DNA polymerase. The primers used in PCR were AAAAGACATGGCGTGGAAAGCCTCTGCAGGC/CGGTCCGAATTCTACTGCCGCAGCTCCAC. The amplified fragments were digested with *Nco*1 and *EcoR*1 and subcloned into PAS2-1 and PACT2 vectors.

Cortactin tagged with six consecutive histidine residues (His-cortactin) was prepared by PCR using the DNA fragments encoding wild-type cortactin as the template. The primers used in PCR were AAGTAAGGATCCTGGAAAGCCTCTGCAGGC/CCCAAAGTCGACTACTGCCGCAGCTCCAC. The amplified fragments were digested with *BamH* 1 and *Sal* 1 and subcloned into pQE-30 vector (Qiagen, Valencia, CA).

Chemical Cross-linking Assay

Recombinant cortactin (3 μg) was initially incubated in 25 μl of 50 mM MOPS (pH7.5) containing 1 mM $MgCl_2$, 1 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and 2 mM N-hydroxysulfosuccinimide (NHS) at room temperature. At 10 min after reaction, the concentrations of EDC and NHS were increased to 2 mM and 4 mM. At 20 min, EDC and NHS were increased further to 3 and 6 mM respectively. At the times of 0, 10, 20 and 40 min after incubation, aliquot reactions were terminated by adding equal volume of 2x SDS sample buffer (125 mM Tris-HCl, pH6.8, containing 4% SDS, 10% 2-mercaptoethanol, 20% glycerol, and 0.004% Bromphenol blue). The reaction samples were resolved by SDS-polyacrylamide gel electrophoresis (PAGE), transferred to a nitrocellulose membrane, and immunoblotted with polyclonal cortactin antibody 2719 (7).

Yeast transformation and β-galactosidase assay

The reporter host strain Y187 (Clontech, Palo Alto, CA) was maintained in YPD medium. The yeast cells were transformed by LiAc-mediated transformation according to the manufacturer's

instruction. The transformants were selected by spreading the transformation suspension on a synthetic dropout medium lacking tryptophan and leucine (SD/-Trp/-Leu). The activity of β-galactosidase (β-gal) was determined by liquid culture assay using o-nitrophenyl β-D-galactopyranoside (ONPG) as substrate, or by whole plate in vivo assay according to the protocol provided by the manufacturer. Briefly, a fresh colony (>2 mm in diameter) was inoculated into 2 ml SD/-Trp/-Leu medium and incubated for overnight. One milliliter of the overnight culture was transferred to a tube containing 4 ml of YPD medium and incubated for 3 to 5 h with shaking. The OD_{600} was recorded when the cells were harvested. The cells from 1.5 ml of culture were pelleted, washed with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1mM $MgSO_4$, pH 7.4), and finally resuspended in 0.3 ml of Z buffer. The yeast suspension was then divided into new tubes at a ratio of 0.1 ml per tube and was stocked in liquid nitrogen. The frozen cells were thawed and mixed with 0.7 ml of Z buffer containing 0.27% of 2-mercaptoethanol. The yeast suspension was then added with 0.16 ml of 4 mg/ml ONPG and incubated at 30°C until the yellow color developed. The reaction was terminated by adding 0.4 ml of 1 M Na_2CO_3. The elapsed time was recorded and the value of OD_{420} was measured. The β-gal activity was calculated according to the formula of 1,000 x OD_{420}/(t x V x OD_{600}), where t = elapsed time in min of incubation; V= 0.1 ml x concentration factor.

In vitro Self-interaction Assays

His-cortactin (2 μg) was incubated with 2 μg of GST-cortactin or GST-cortactin variants in 100 mM Tris-HCl, pH7.5, containing 4 M guanidine and 10 mM DTT at room temperature for 5 min. The denatured proteins were diluted to 1 ml with 20 mM Tris-Cl, pH7.5, containing 100 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 0.1% Triton X-100, 25 mM imdazole and 3 mg/ml bovine serum albumin. The diluted samples were mixed with 30 μl of Ni-NTA agarose and rotated at 4°C for 2 h. After a brief precipitation, the supernatant was removed and the agarose beads were resuspended in 40 μl of 2xSDS sample buffer. The samples were analyzed by SDS-PAGE and were transferred to a nitrocellulose membrane. The GST-cortactin proteins were detected by blotting with an anti-GST monoclonal antibody (upstate Biotechnology Inc., Lake Placid, NY).

Preparation of recombinant cortactin and its mutant

GST fusion proteins were prepared, digested with thrombin and purified as GST-free protein as previously described (13). His-cortactin was expressed in E. coli strain M15 pREP24 (Qiagen, Valencia, CA). The expression of the protein was induced by 1 mM IPTG, and purified by Ni-NTA affinity chromatography. The concentrations of proteins were determined by the Dc protein assay kit (Bio-Rad, Hercules, CA) according to the instruction of the manufacturer.

F-actin Cross-linking and F-actin binding assay

The F-actin cross-linking activity of wild-type cortactin and its mutant was measured as described previously (13). To analyze the affinity for F-actin binding, wild-type cortactin or mutant $Cort_{D421D466D482D485}$ at different concentrations were incubated with F-actin (4μM) in 100 μl of TKM buffer (13) for 60 min. The mixtures were then centrifuged at 170,000xg for 30 min. The amounts of cortactin (or mutant $Cort_{D421D466D482D485}$) in the supernatants and the pellets were analyzed by SDS-PAGE, followed by immunoblotting with monoclonal cortactin antibody 4F11 (Clontech). The amounts of free- and bound-cortactin (or cortactin mutant) were quantitated by density analysis using NIH Image.

RESULTS AND DISCUSSION:

Oligomerization of cortactin was initially examined by a chemical cross-linking assay using a zero-length cross-linker EDC. As shown in Fig. 1A, recombinant cortactin normally exhibits its characteristic double bands of 80 and 85 kDa in a denatured SDS-PAGE. Once EDC was added, these two bands were gradually shifted from 80/85 to a position of 160 kDa, indicating a dimerized form of cortactin present in the solution.

To further verify the dimerization of cortactin, cortactin was constructed as a fusion protein either to GST epitope or to 6xHis epitope. The interaction of GST-cortactin with His-cortactin was then analyzed by co-precipitation with Ni-NTA agarose, which specifically recognizes the 6xHis epitope. While few GST cortactin proteins could be precipitated by Ni-NTA agarose in the absence of His-

cortactin, the presence of His-cortactin significantly enhanced the precipitation of GST-cortactin, indicating an intermolecular interaction between these two forms of cortactin (Fig 1B).

The self-interaction of cortactin was also examined in a yeast two-hybrid system. A c-DNA encoding the full-length murine cortactin was constructed into plasmids PAS2-1 (GAL4 DNA binding domain vector) and PACT2 (GAL4 transcription activation domain vector). The resulting plasmids, PAS2-1-Cort and PACT2-Cort, were co-transformed into yeast and the interaction of cortactin and cortactin was measured by the enzymatic activity of β-gal within yeast transformants. As shown in Fig.1C, co-transformation of PAS2-1-Cort with PACT2-Cort resulted in a dramatic increase in the β-gal activity, indicating an intermolecular interaction between cortactin proteins encoded by the two plasmids. The increase in the activity of β-gal was unlikely due to a non-specific reaction between cortactin and GAL4 proteins because neither co-transformation of PAS2-1 with PACT2-Cort nor co-transformation of PAS2-1-Cort with PACT2 induced significantly the β-gal activity (Fig 1C).

To determine whether the cortactin dimerization is the mechanism for its apparent F-actin cross-linking activity, we studied a cortactin mutant, $Cort_{D421D466D482D485}$, where the tyrosine residues (Tyr-421, Tyr-466, Tyr-482, and Tyr-485) targeted by Src in vitro were substituted with aspartic acids. The rationale for this substitution was that aspartic acid shares a negative charge as phosphorylated tyrosine and the resulting mutant might act similarly as tyrosine phosphorylated cortactin, as found in other proteins (17). Indeed, the mutant showed a 80% lower activity for F-actin cross-linking than wild-type cortactin as analyzed by either sedimentation analysis or by confocal laser microscopic analysis (Fig. 2A). This attenuated activity is also in concert with our previous report that Src can inhibit as much as 80% of the F-actin cross-linking activity of cortactin (13).

The reduced activity for the F-actin cross-linking exhibited by the mutant $Cort_{D421D466D482D485}$ was not due to a reduced affinity for F-actin binding because the mutant maintains a similar or higher activity for F-actin binding (Fig 2B). Interestingly, the mutant fused with GST showed little affinity for His-cortactin (Fig 3A). In addition, a cortactin mutant where Tyr421 and Tyr466 were replaced by aspartic acids also

failed to interact with each other within yeast (Fig 3B). Thus, cortactin dimerization is correlated with its activity for F-actin cross-linking.

The role of tyrosine phosphorylation in the regulation of cortactin dimerization was further analyzed by examining the effect of Src on the dimerization of cortactin in the in vitro self-interaction assay. As shown in Fig 4, the ability of GST-cortactin to co-precipitate with His-cortactin was significantly inhibited by Src.

Based on these findings, we propose a model for the regulation of the function of cortactin by tyrosine phosphorylation. Cortactin normally exists as a dimer and cross-links F-actin via its dimerization. Upon tyrosine phosphorylation, however, the cortactin homodimer is disrupted, resulting in a reduced activity for F-actin cross-linking. The significance of this model is that it provides a novel signal pathway from extracellular stimuli to the cytoskeletal reorganization via Src tyrosine kinase. Because cortactin co-localizes with F-actin primarily within lamellipodia and punctate-like membrane structures (6;10), its flexible activity for F-actin cross-linking contributes likely to the flexibility of these structures, which is essential for cell migration and cell shape change. Furthermore, cortactin and Src are widely expressed in adherent cells and tyrosine phosphorylation of cortactin appears to be a common event implicated in many extracellular stimuli. This signal pathway may represent a general mechanism for the cytoskeletal reorganization. Indeed, we have previously shown that overexpression of a cortactin mutant with defect in tyrosine phosphorylation can cause a decrease in cell migration (10). Furthermore, a poor tyrosine phosphorylation of cortactin is correlated with a poor migratory response to FGF-1 within the cells lacking of Src (12).

Dimerization is a common mechanism for many other F-actin cross-linkers, including α-actinin (18), ABP-280 (16), spectrin (15). Unlike these F-actin cross-linkers, however, cortactin has its unique F-actin binding sequence that is composed of six copies of 37-amino-acid repeat. The existing data suggest that the repeat domain constitute only a single F-actin binding site because the F-actin binding requires at least four repeat units. Furthermore, a mutant deleted the C-terminal half including tyrosine residues

targeted by Src is unable to form a dimer, as assayed by the two-hybrid yeast assay (Huang, unpublished result), indicating that the C-terminal half domain is important for cortactin dimerization. While the mechanism for cortactin dimerization remains unknown, it is likely that tyrosine phosphorylation at these residues induces a conformational change. This subsequently causes disruption of the cortactin homo-dimer. Characterization of the detailed structure domains or sequences for cortactin dimerization in the future will permit to dissect the mechanism how tyrosine phosphorylation can affect the cortactin dimerization.

FIGUREE LEGENDS:

Fig. 1. **A**. Chemical cross-linking of cortactin. Recombinant cortactin was incubated with EDC and NHS as described in Experimental Procedures. At the times indicated, aliquots of samples were terminated and analyzed with SDS-PAGE followed by immunoblotting with cortactin antibody 2719. **B**. In vitro self-interaction of cortactin. The co-precipitation of GST cortactin with His-cortactin was analyzed as described in experimental Procedures. **C**. Cortactin dimerization analyzed by yeast two-hybrid system. The plasmids encoding murine cortactin were co-transformed into yeast with the following combinations: 1, PAS2-1/PACT2; 2, PAS2-1-CORT/PACT2; 3, PAS2-1/PACT2-CORT; and 4, PAS2-1-CORT/PACT2-CORT. The β-gal activity was determined by liquid culture assay using ONPG as substrates as described in the Experimental Procedures.

Fig. 2. The mutant $Cort_{D421D466D482D485}$ has reduced activity for F-actin cross-linking while maintaining F-actin binding activity. **A**. The F-actin cross-linking activities of cortactin were attenuated by substituting the tyrosine residues with aspartic acid residues. Upper panel, wild-type cortactin (O) and $Cort_{D421D466D482D485}$ (□) were analyzed for F-actin cross-linking using co-sedimentation analysis as described in the Experimental Procedure. The data represents the average of three independent experiments. Lower panel, fluorescein-labeled F-actin (2 μM) was incubated with 400 nM wild-type cortactin (b), 400 nM $Cort_{D421D466D482D485}$ (c) or the buffer for proteins (a) for 1 h at room temperature. The cross-linked actin

filaments were visualized by confocal microscopy. **B.** The relative binding activity of wild-type cortactin and mutant $Cort_{D421D466D482D485}$ for F-actin were analyzed as described in Experimental Procedures.

Fig. 3. Substituting Src targeted tyrosine residues with aspartic acids impaired the self-interaction of cortactin. **A.** GST-cortactin or GST-$Cort_{D421D466D482D485}$ was incubated with His-cortactin at 4°C for 2 h. Co-precipitation of GST-cortactin variants with His-cortactin was analyzed. **B.** The plasmids encoding wild-type cortactin and mutant $Cort_{D421D466}$ were co-transformed into yeast with the combination as indicated. The β-gal activities of the transformants were used as the indication of protein-protein interaction.

Fig. 4. Src inhibits the self-interaction of cortactin. GST-cortactin was phosphorylated with or without Src (500nM) for 2 h at room temperature, and then incubated with His-cortactin at 4°C for 2 h. The co-precipitation was analyzed as described in Experimental Procedures.

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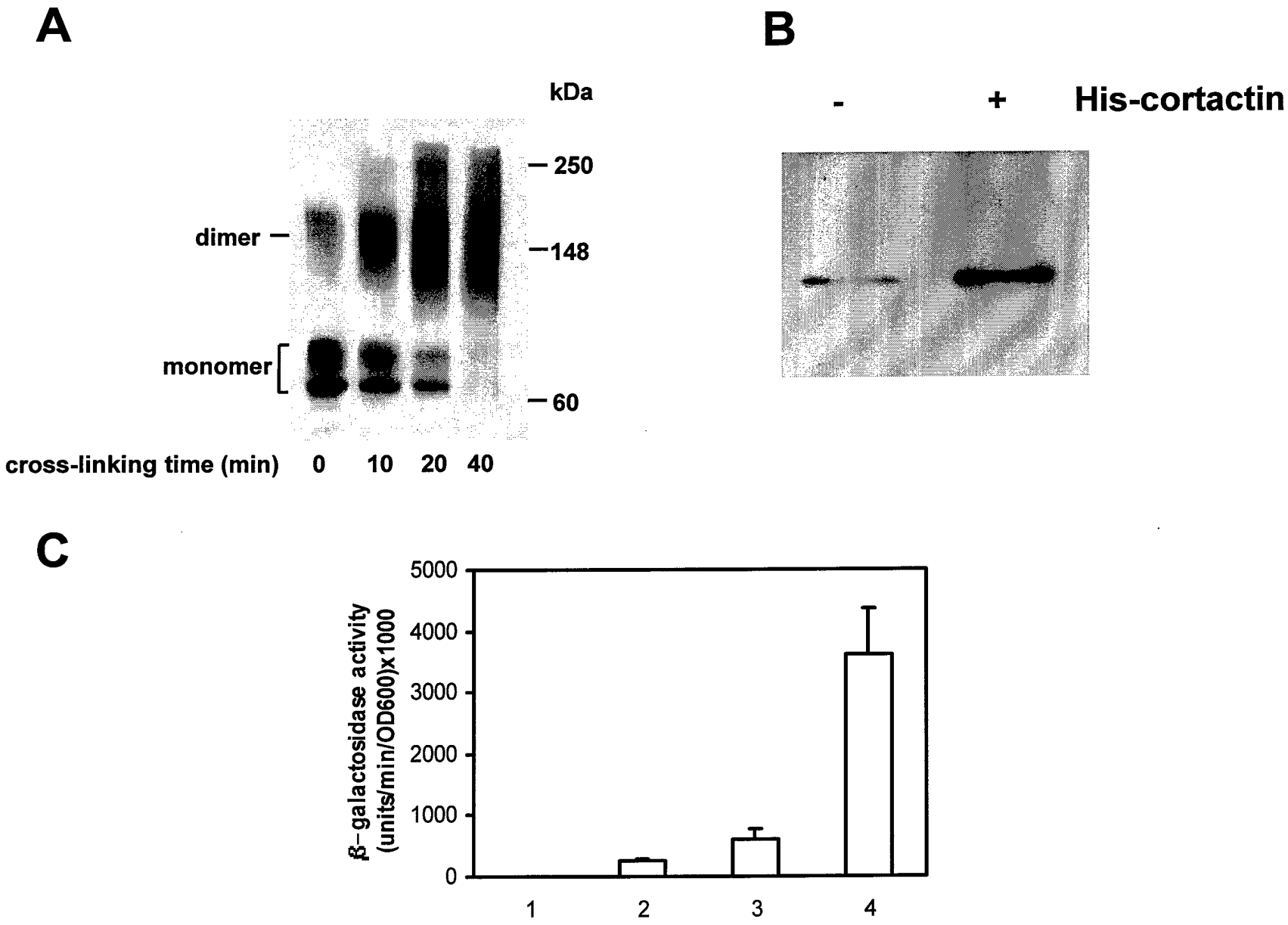

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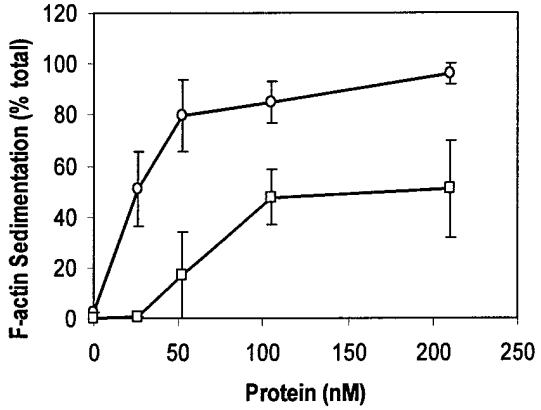

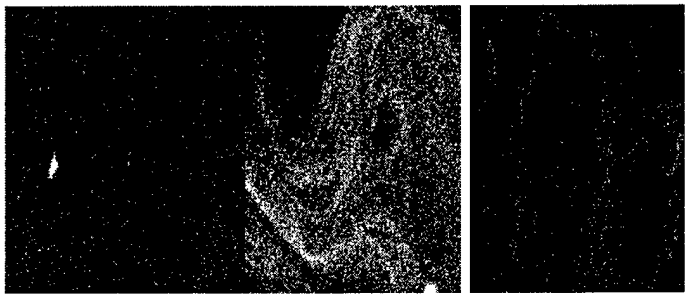

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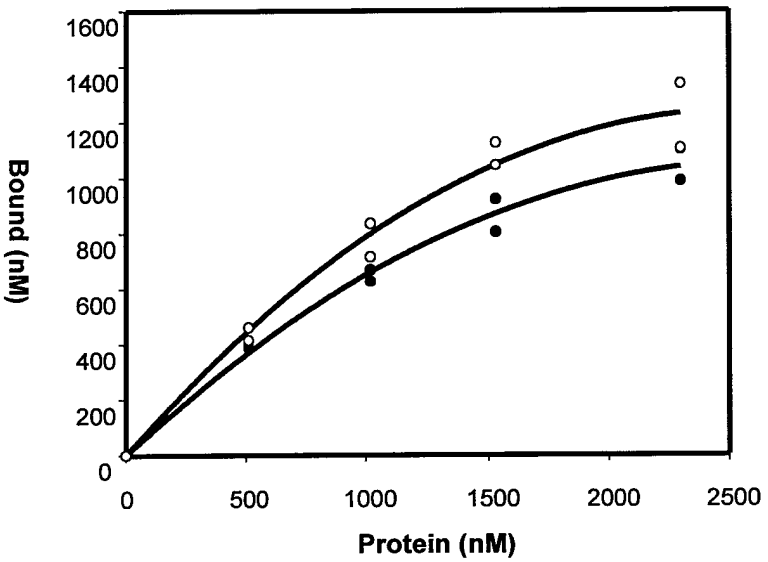

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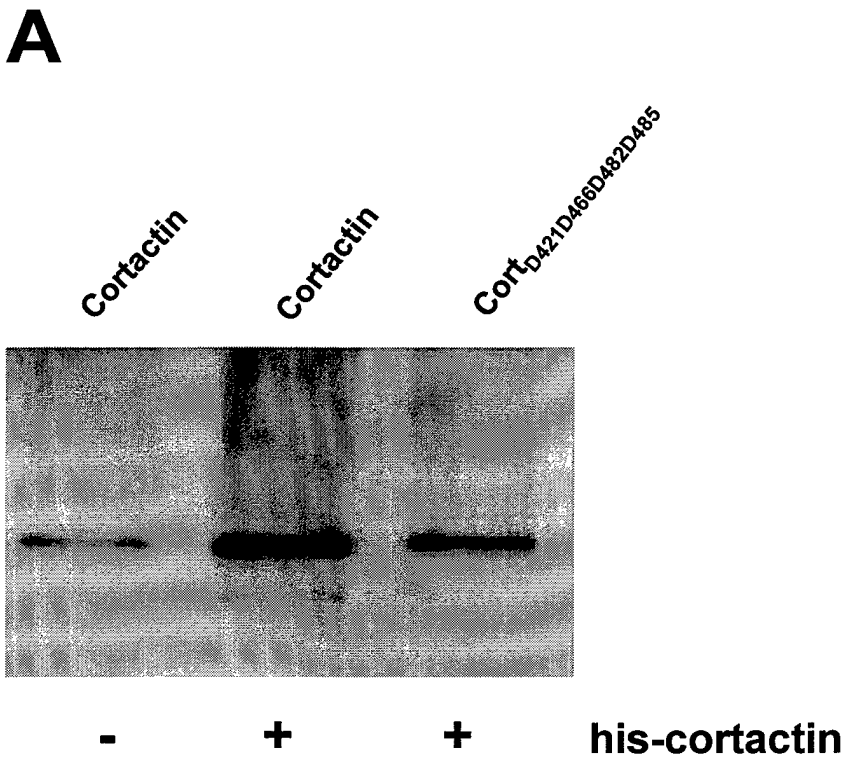

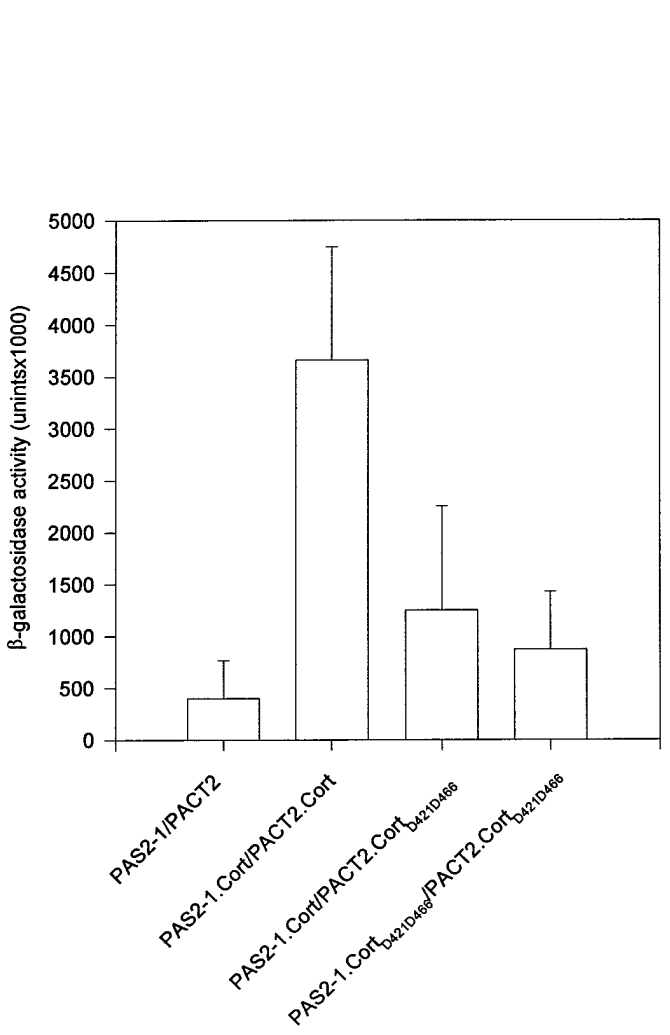

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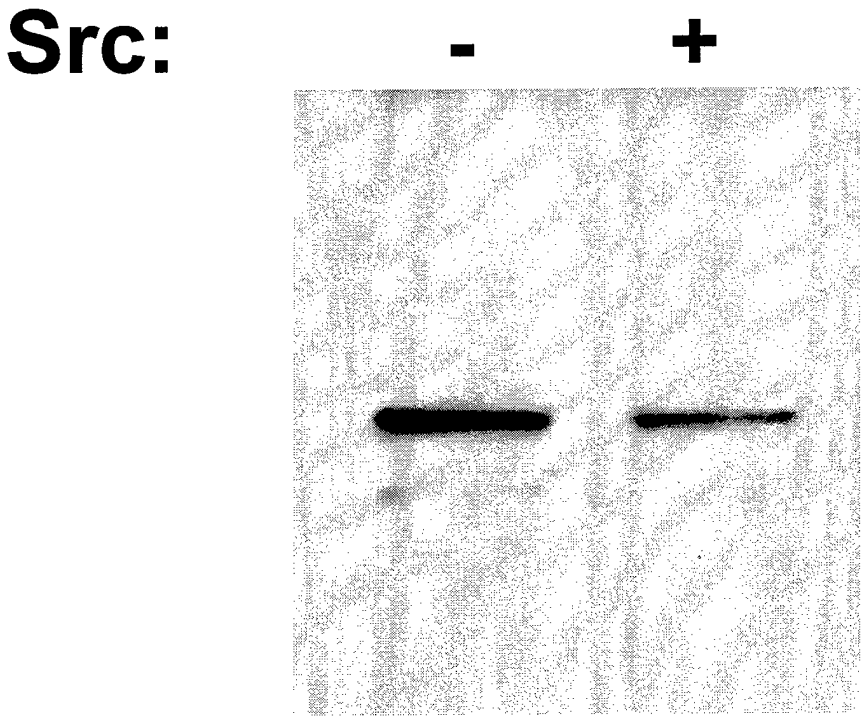

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Src is required for cell migration and shape changes induced by fibroblast growth factor 1

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Fibroblast growth factor 1 (FGF-1) is a potent chemotactic factor and induces tyrosine phosphorylation of a cortical actin-associated protein (cortactin). The tyrosine phosphorylation of cortactin induced by FGF-1 requires the tyrosine residues 421, 482 and 466, which are targeted by the protein tyrosine kinase Src *in vitro*. Furthermore, FGF-1 is unable to induce tyrosine phosphorylation of cortactin within the cells derived from Src knockout mice ($Src^{-/-}$), indicating that Src is required for the tyrosine phosphorylation of cortactin induced by FGF-1. Although $Src^{-/-}$ cells are able to undergo rapid proliferation, they are impaired to respond to FGF-1 for the shape change and cell migration. Morphological analysis further reveals that FGF-1 fails to induce the formation of polarized lamellipodia and the translocation of cortactin into the leading edge of $Src^{-/-}$ cells. Consistent with the mitogenic response to FGF-1, the lack of Src does not affect the tyrosine phosphorylation of Snt (or Frs2), a FGF-1 early signaling protein that links to Ras. Therefore, our data support the notion that Src and cortactin participate in a FGF signal pathway for cell migration and shape change rather than mitogenesis.

Keywords:

Introduction

The family of fibroblast growth factor (FGF) represents a large group of polypeptides that play important roles in embryonic development, tissue growth, wound healing, and angiogenesis (Burgess *et al.*, 1989). The prototypes of FGF, namely FGF-1 and FGF-2, are mitogenic and chemotactic for many mesenchyme-derived cells (Gospodarowicz, 1974; Maciag *et al.*, 1982). During the early response to FGF-1 or FGF-2, several intracellular proteins are phosphorylated at tyrosine residues (Huang *et al.*, 1986). These phosphotyrosyl proteins include phospholipase C γ (PLC-γ), Snt (or Frs2), Shc, Gab-1, and MAP kinases (Bogoyevitch *et al.*, 1994; Burgess *et al.*, 1990; Kouhara *et al.*, 1997; Ong *et al.*, 1997; Wang *et al.*, 1994, 1996). Whereas PLC-γ is important for FGF-mediated phosphatidylinositol turnover, it is not necessary for FGF-induced mitogenesis (Mohammadi *et al.*, 1992; Peters *et al.*, 1992). Snt encodes a protein that has an electrophoretic motility around 90 kDa (Kouhara *et al.*, 1997) and binds to FGF receptor 1 (FGFR-1) through its phosphotyrosine binding domain in a phosphotyrosine independent manner (Xu *et al.*, 1998). Snt functions as a lipid-anchored docking protein and targets the signaling adaptor Grb-2, either directly or through Sph-2 protein tyrosine phosphatase, to the plasma membrane (Hadari *et al.*, 1998; Kouhara *et al.*, 1997). Because Grb-2 binds to Sos, a Ras guanine nucleotide exchange factor (Buday *et al.*, 1993; Egan *et al.*, 1993; Rozakis-Adcock *et al.*, 1993), Snt is likely to be a primary component involved in the activation of the complexes of Ras/Raf/mitogen-activated protein kinase (MAPK), the major signal pathway from the plasma membrane to the nucleus. Likewise, Gab-1 (an IRS-related protein) and Shc act also as signaling adaptors and are likely involved in the similar pathway to the activation of Ras (Holgado-Madruga *et al.*, 1996; Rozakis-Adcock *et al.*, 1992). Although the exact roles of these molecules in the biological function of FGF are not fully elucidated, it is assumed that these factors may ultimately be important for the FGF's mitogenic activity.

It is less understood, however, how FGFs signal cell shape changes and migration. We have previously shown that FGF-1 requires a prolonged stimulation to reach a maximal mitogenic response (Zhan *et al.*, 1993a). Analysis of this late response (4–12 h) has revealed a series of tyrosine phosphorylated proteins that are not apparent during the early response. One of these proteins was characterized as cortactin, a prominent substrate of Src protein tyrosine kinase (Zhan *et al.*, 1993b). Cortactin is also a potent cross-linker for filamentous actin (F-actin) (Huang *et al.*, 1997) and co-localizes with F-actin in cell peripheral areas and punctate-like membrane structures (Wu *et al.*, 1993). We have recently described that the F-actin cross-linking activity of cortactin can be attenuated by Src-mediated tyrosine phosphorylation (Huang *et al.*, 1997). Furthermore, overexpression of a cortactin mutated at tyrosine phosphorylation sites impairs the migration of endothelial cells (Huang *et al.*, 1998). Therefore, these data indicate that cortactin acts as a modulator for the actin cytoskeleton.

The evidence for the role of Src in FGF signaling also begins emerging. In a previous investigation of FGF signaling, we described that the activated FGFR-1 is able to transiently associate with Src-like proteins in a manner dependent on tyrosine phosphorylation of FGFR-1 and the SH2 domain of Src (Zhan *et al.*, 1994). Overexpression of a normal Src can increase the response to FGF-1 mediated cellular scattering and motility (Rodier *et al.*, 1995). On the other hand,

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scattering response without affecting mitogenesis induced by FGF-1 or EGF. In addition, the early withdrawal of FGF-1 from culture medium can induce cell migration without promoting DNA synthesis, this property is apparently correlated with a transient increase in Src kinase activity (LaVallee *et al.*, 1998). While these studies suggest that a Src-related activity may be involved in a FGF signaling independent on mitogenesis, neither the specific Src family members nor particular Src substrates have been defined. Furthermore, direct genetic evidence for the role of Src in the growth factor induced cell migration is still missing. In this report, we examined whether Src is required for the FGF-1 mediated tyrosine phosphorylation of cortactin within the cells derived from Src knockout mice. We found that a cortactin mutated at the tyrosine residues that are targeted by Src is impaired to respond to FGF-1 stimulation. In addition, cells lacking the Src gene are able to undergo proliferation in the presence of FGF-1, though these cells show fewer changes in morphology and have slower motility compared to normal cells. Furthermore, FGF-1 is able to induce tyrosine phosphorylation of Snt but not cortactin in Src knockout cells. These findings demonstrate that Src is the major kinase responsible for FGF-1-induced tyrosine phosphorylation of cortactin, and Src and cortactin are implicated in a signal pathway for cell migration and shape change, which is apparently distinct from that involved in the mitogenic response.

Results

Src is essential for the tyrosine phosphorylation of cortactin induced by FGF-1

In the attempt to verify that Src is required for the tyrosine phosphorylation of cortactin induced by FGF-1, we initially evaluated whether the tyrosine residues targeted by Src are necessary for FGF-1 mediated phosphorylation. In a previous study, we determined that the primary tyrosine residues targeted by Src reside at Tyr-421, Tyr-466 and Tyr-482 (Huang *et al.*, 1998). Thus, we analysed NIH3T3 cells expressing *myc*-Cort$_{F421F466F482}$, a cortactin mutant in which the three tyrosine residues are replaced with phenylalanines and the NH2-terminus is fused to the *myc* epitope (Huang *et al.*, 1998). As a control, a wild-type murine cortactin fused to the same *myc* epitope (*myc*-cortactin) was analysed in parallel. As shown in Figure 1, the levels of tyrosine phosphorylated *myc*-cortactin and the endogenous cortactin significantly increased within 12 h after FGF-1 stimulation, although the gel motility of *myc*-cortactin fusion was slightly slower than that of the endogenous cortactin, presumably due to the *myc* tag. In contrast, the mutant Cort$_{F421F466F482}$ failed to increase in its tyrosine phosphorylation in response to FGF-1. This result indicates that the tyrosine phosphorylation of cortactin induced by FGF-1 occurs at the same residues that are targeted by Src.

To further confirm the role of Src in the tyrosine phosphorylation of cortactin, we examined embryonic fibroblasts derived from the Src-knockout mice (Src$^{-/-}$) and fibroblasts derived from a normal mouse embryo (Src$^{+/+}$). The cells were maintained at near confluence in a serum-free defined medium supplemented with insulin (DMI) for 48 h and subsequently stimulated with FGF-1 for various times. The total cell lysates were prepared and analysed by immunophosphotyrosine blot analysis. It appeared that Snt, which is a major FGF responsive protein and migrates at 90 kDa,

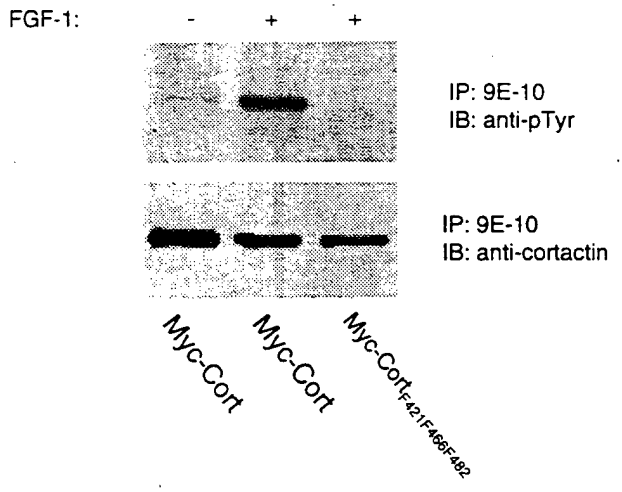

Figure 1 Tyrosine phosphorylation of cortactin induced by FGF-1 requires the tyrosine residues targeted by Src. Quiescent NIH3T3 cells expressing myc-Cort and myc-Cort$_{F421F466F466}$ were stimulated with FGF-1 for 12 h. The lysates from the stimulated cells were immunoprecipitated with a monoclonal cortactin antibody 9E-10 and further immunoblotted with a polyclonal phosphotyrosine antibody. The same blot was stripped and re-

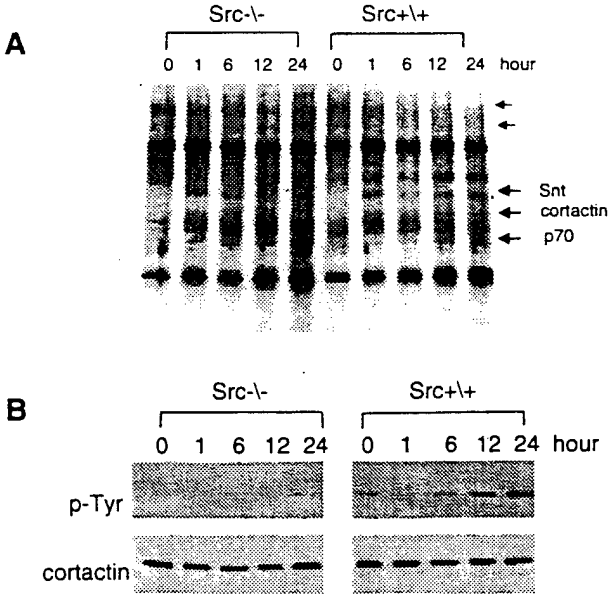

Figure 2 Tyrosine phosphorylation of cortactin in Src$^{-/-}$ cells. (a) Quiescent Src$^{-/-}$ and Src$^{+/+}$ cells were treated with FGF-1 for the times indicated. Total cell lysates were prepared by dissolving stimulated cells in SDS sample buffer. The lysates were then separated in SDS–PAGE and further blotted with monoclonal anti-phosphotyrosine antibody (4G10) as described in Materials and methods. (b) FGF-1 stimulated cell lysates were immunoprecipitated with a polyclonal cortactin antibody. The precipitates were immunoblotted with monoclonal anti-phospho-

was induced similarly in both types of cells (Figure 2a). However, a phosphorylated band around 80 kDa was detected only in $Src^{+/+}$ but not in $Src^{-/-}$ cells (Figure 2a). The p80 band apparently co-migrated with cortactin (data not shown), which was further confirmed by immunoprecipitation with a cortactin antibody followed by immunophosphotyrosine blot analysis. As shown in Figure 2b, cortactin was hyperphosphorylated significantly in $Src^{+/+}$ cells after 12 h of FGF-1 stimulation. In contrast, it was weakly phosphorylated within $Src^{-/-}$ cells, although there was a slight increase in tyrosine phosphorylation of cortactin at 24 h of stimulation. This slight increase observed at 24 h was apparently due to a differential sample loading as indicated by analysing the same blot using a cortactin antibody (Figure 2b, the lower panel). Interestingly, we also observed several other phosphotyrosyl bands including one of 70 kDa and two bands between 130–200 kDa, which were actually enhanced in the FGF-1 stimulated $Src^{-/-}$ cells (Figure 2a). However, the identities of these bands are currently unknown. Taken together, these data demonstrated that Src is likely the primary kinase for FGF-induced phosphorylation of cortactin, and cortactin is apparently one of the major phosphotyrosyl proteins induced by FGF-1 in a Src-dependent manner.

Src is required for FGF-1-mediated cell shape change and migration

To elucidate the role of Src in the function of FGF-1, we also examined the morphological response of $Src^{-/-}$ and $Src^{+/+}$ to FGF-1. Under serum-starved culture conditions, both types of cells exhibited a typical quiescent morphology characterized by a flat monolayer (Figure 3). When the cells were exposed to FGF-

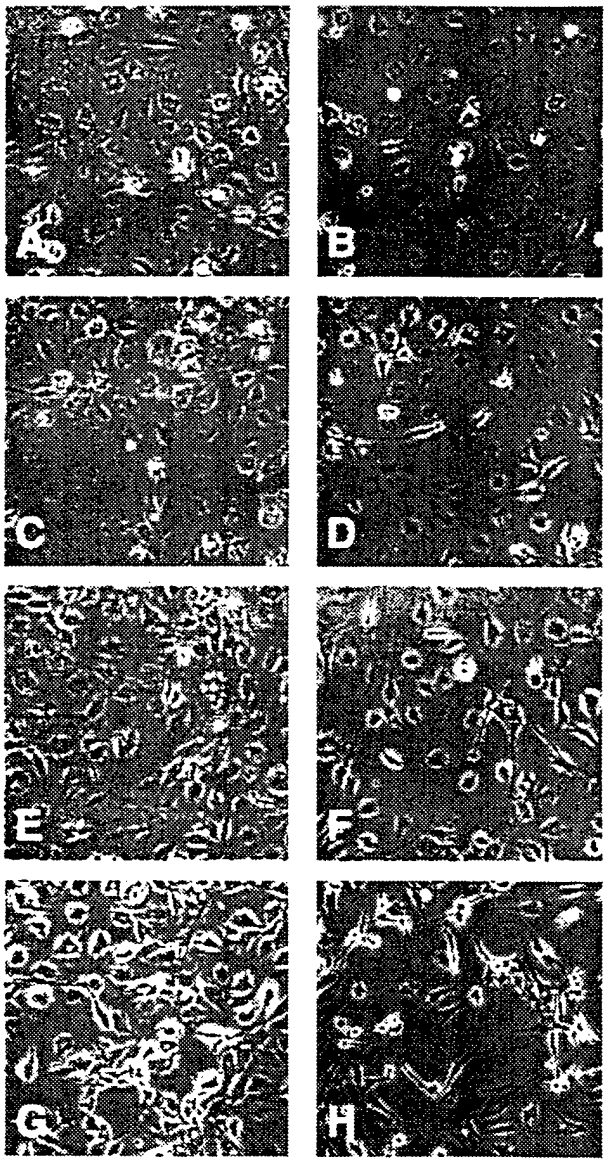

Figure 3 FGF-1 induces different morphological changes in $Src^{-/-}$ and $Src^{+/+}$ cells. Quiescent $Src^{-/-}$ (**a**, **c**, **e** and **g**) and $Src^{+/+}$ (**b**, **d**, **f** and **h**) cells were stimulated with FGF-1 for [illegible]

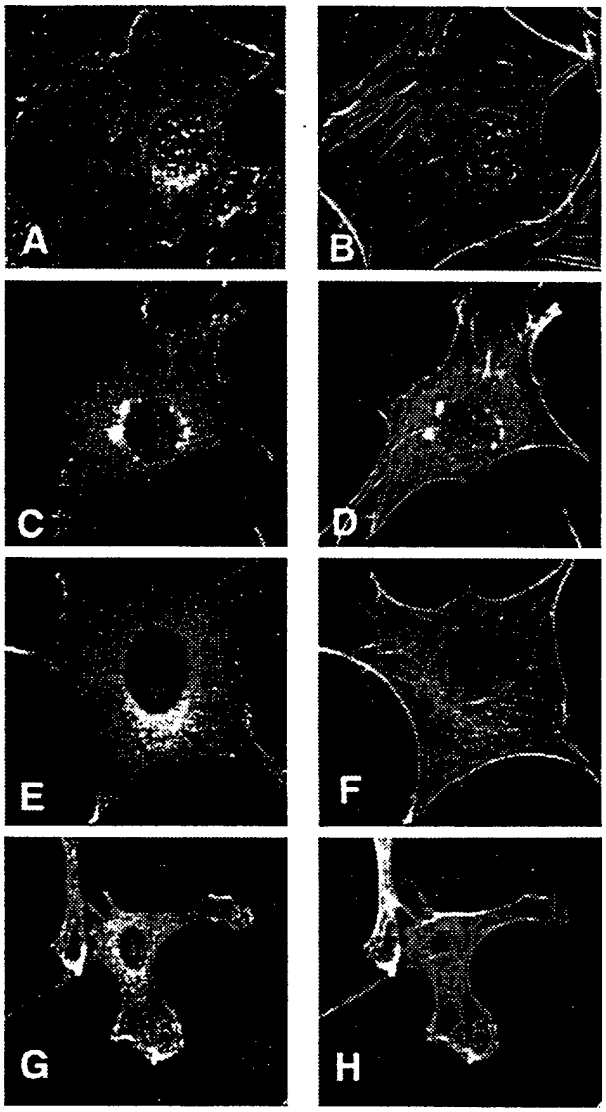

Figure 4 Redistribution of cortactin within $Src^{-/-}$ cells is impaired. Quiescent $Src^{-/-}$ (**a**–**d**) and $Src^{+/+}$ cells (**e** ~~to~~ **h**) were stimulated with or without FGF-1 for 15 h (**a**, **b**, **e** and **f**: untreated; **c**, **d**, **g** and **h**: FGF-1 treated). The cells were then double-stained with the polyclonal cortactin antibody C001 (**a**, **c**, **e** and **g**) and FITC-phalloidin (**b**, **d**, **f** and **h**) as described in Materials and methods. The stained cells were examined under

1 for 12 h, $Src^{-/-}$ cells underwent a significant shape change typified by a greatly elongated cytoplasm (Figure 3). However, this change was not observed when $Src^{-/-}$ cells were analysed, although many of these cells found up after 12 h of FGF-1 stimulation. A close examination by confocal laser scanning microscopy also revealed a differential cortactin staining between these two types of cells. While cortactin was found within the areas around the periphery, the perinucleus, and punctate-like structures within the cytoplasm of both quiescent cells (Figure 4a,e), cortactin and F-actin co-stained abundantly within polarized lamellipodia, as defined by large intervening regions of the $Src^{-/-}$ cells at 12 h stimulation (Figure 4g,h). Concomitant with the association of more cortactin proteins with these polarized leading structures, less cortactin staining was detected in the perinucleus of FGF-1 treated $Src^{-/-}$ cells. In contrast, the Src cells treated with FGF-1 did not show significantly polarized lamellipodia associated with cortactin and F-actin (Figure 4c,d). Instead, cortactin was more enriched at the peri-nucleus within those cells (Figure 4c).

The differential response of these cells to FGF-1 with respect to the morphological changes was apparently correlated with their motility as analysed by a wound-healing assay. As shown in Figure 5, both Src and $Src^{-/-}$ cells did not show apparent migration within 6 h into the wounded areas generated by a rubber policeman when FGF-1 was absent (Figure 5a,c). In the presence of FGF-1, however, $Src^{-/-}$ cells displayed extensive elongation of their cytoplasm. Concomitant with the elongated morphology, a significant number of $Src^{-/-}$ cells was found in the wounded area (Figure 5d). In contrast, few $Src^{-/-}$ cells showed migration, although many of them exhibited rounding-up morphology (Figure 5b).

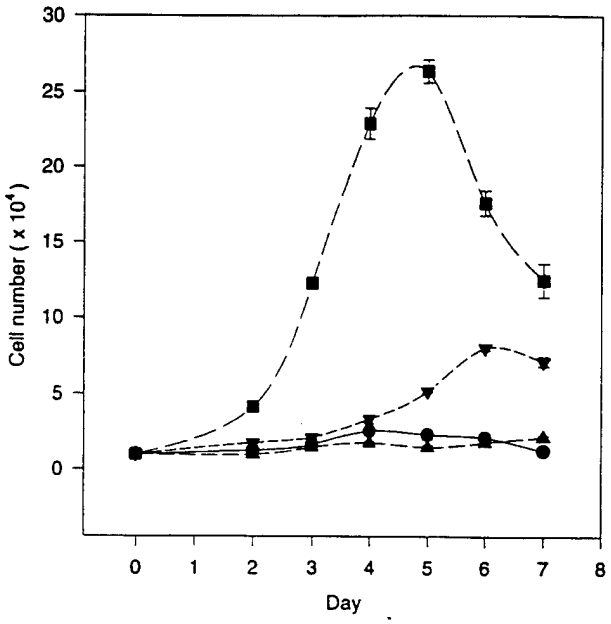

Figure 6 The lack of Src does not affect cell proliferation. $Src^{-/-}$ and $Src^{+/+}$ cells were seeded at 1×10^4 cells per well in a 24-well plate. At 8 h after plating, the culture medium was changed to DMI supplemented with FGF-1 (10 ng/ml) and heparin (10 μ/ml). In the control samples, cells were maintained in DMI. At the times indicated, cells were trypsinized and counted using a hemocytometer. Each time point represents a mean of three independent experiments. Labels: ●, $Src^{-/-}$ in DMI only; ■, $Src^{-/-}$ in DMI plus FGF-1; ▲, $Src^{+/+}$ in DMI; and ▼, $Src^{+/+}$ in DMI plus FGF-1

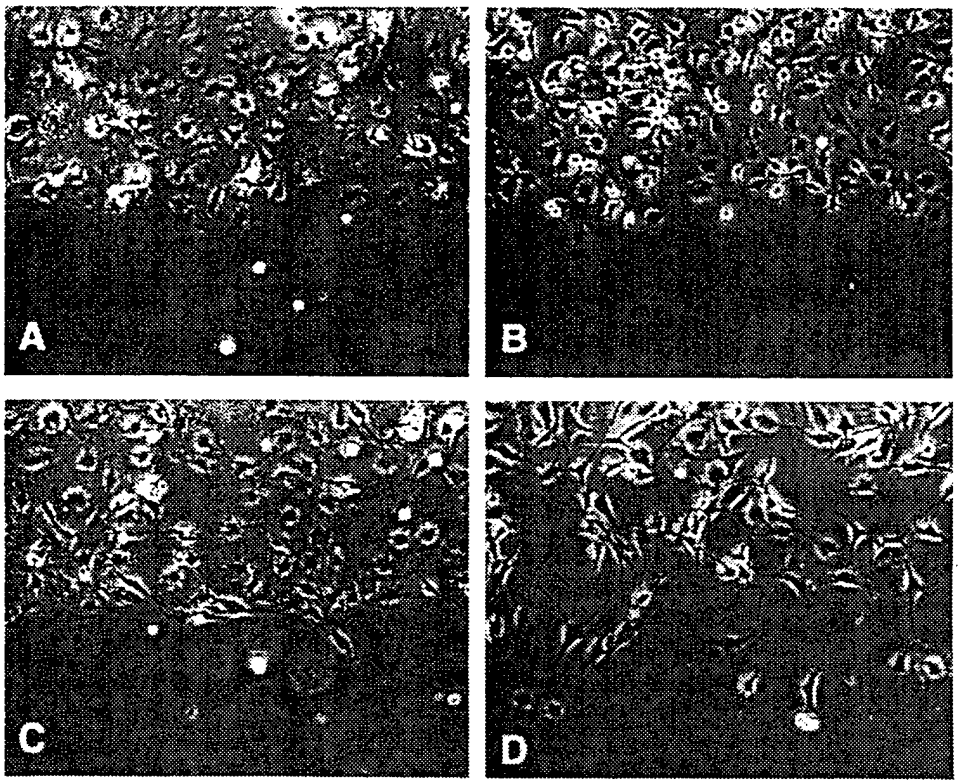

Figure 5 The lack of Src impairs cell migration. Quiescent $Src^{-/-}$ (**a** and **b**) and $Src^{+/+}$ cells (**c** and **d**) were stimulated with (**b** and

Src is not required for FGF-1 mediated cell proliferation

The rounding-up morphology exhibited by Src^{-} cells in the presence of FGF-1 suggested that those cells were able to undergo mitosis. To further confirm this notion, we have measured their proliferative response to FGF-1. Both Src$^{+/-}$ and Src^{-} cells were plated at approximately 5% confluence and maintained in DMI supplemented with FGF-1. As shown in Figure 6, both types of cells grew in a FGF-1-dependent manner. Interestingly, Src$^{-/-}$ cells appeared to grow even faster than Src$^{+/-}$ cells. The maximal number of Src^{-} cells in the presence of FGF-1 was nearly four times higher than that of Src$^{+/-}$ cells (Figure 6). Thus, the lack of Src apparently does not inhibit but actually enhances the response to FGF-1 for proliferation.

Discussion

In this study, we provided evidence that Src is required for FGF-1-mediated shape change and cell migration. Our conclusion further supports the notion that Src plays a vital role in the signaling pathways for cell migration and shape change triggered by growth factors. However, our data also demonstrate that Src is not required for the FGF-1 mediated cell proliferation. In fact, the cells lacking Src grow much faster than normal cells (Figure 6). Consistent with the mitogenic response to FGF-1, the lack of Src does not impair the tyrosine phosphorylation of Snt (or Frs2). Snt has been shown to be a direct substrate for FGF receptors (Xu *et al.*, 1998). Because Snt shares homologous sequence with insulin receptor substrate 1, and binds to Grb-2 proteins, it is likely a major signaling molecule linking to the Ras/MAPK pathway and is essential for FGF's mitogenic activity. Thus, it appears that Src and cortactin are involved in a discrete signal pathway that primarily regulates the cytoskeletal changes. In support of this notion, transient FGF stimulation does not induce a maximum DNA synthesis but is able to induce the activation of Src and tyrosine phosphorylation of cortactin (LaVallee *et al.*, 1998). Furthermore, it is known that oncogenic Src (v-Src) transforms cells in a manner independent of MAPK, a key component for its mitogenic activity (Oldham *et al.*, 1998).

In light of the presence of many members in the Src family and other non-receptor tyrosine kinases (Thomas *et al.*, 1997), FGF-1 could utilize kinases other than Src to phosphorylate these molecules, as indicated by a recent report that FER, a non Src-related intracellular tyrosine kinases, is involved in a PDGF-mediated cortactin phosphorylation (Kim *et al.*, 1998). However, we found that the tyrosine phosphorylation of cortactin induced by FGF-1 is significantly impaired in the cells deficient in Src alone. Furthermore, the tyrosine residues targeted by Src are the same as those mediated by FGF-1 (Figure 1). These results reinforce the notion that cortactin is a preferred substate for Src compared to other Src-related kinases (Thomas *et al.*, 1995). It is also noteworthy that cortactin is the major tyrosine phosphorylated protein that is impaired in FGF-1-induced Src^{-} cells (Figure

Cortactin is a cell periphery-associated protein that binds to and cross-links filamentous actin (Huang *et al.*, 1997; Zhan *et al.*, 1993b). In quiescent Src^{+} or Src^{-} cells, cortactin localizes evenly around the cell margins. This is consistent with our previous report that a mutant lacking tyrosine phosphorylation sites is still able to associate with cell peripheral structures in endothelial cells (Huang *et al.*, 1998). However, in FGF-stimulated Src^{-} cells, the majority of cortactin was accumulated in the leading edges of the cells. This accumulation was apparently impaired within Src^{-} cells, where cortactin is more concentrated around the nucleus. Thus, it appears that Src is required for the dynamic movement of cortactin within the cytoplasm. Interestingly, the rearranged cortactin co-localizes with F-actin either in newly formed lamellipodia in normal cells (Figure 4h) or around the nucleus (Figure 4d) in Src^{-} cells, indicating that the movement of cortactin within cells is coupled with F-actin. A recent report has described that Rac is involved in the translocation of cortactin in PDGF-stimulated cells (Weed *et al.*, 1998). While Src can reportedly activate Ras-like GTPase (Chang *et al.*, 1995; Schieffer *et al.*, 1996), the lack of Src is unlikely to affect the activation of Rac in FGF treated cells because Src is not required for the activation of Snt, a primary signaling molecule that links to Ras, which in turn can activate Rac (Hall, 1998). Alternatively, the dynamic movement of cortactin requires its tyrosine phosphorylation, which apparently attenuates its F-actin cross-linking activity (Huang *et al.*, 1997). It has been suggested that the movement of cellular particles is due to a retrograde actin flow, a movement of newly formed actin filaments from the front of lamellipodia to the opposite direction of cell forward (Cramer, 1997). One model for the driving actin retrograde flow is that disassociation of cross-linked actin network at the back of the lamellipodium will generate a tension gradient which can drive retrograde flow of actin (Mogilner *et al.*, 1996). Thus, it will be of interest to define whether cortactin is involved in a driving force for actin retrograde flow. Further experiments using mutants for each of these pathways should dissect the mechanism for the cortactin translocation induced by FGF-1.

Materials and methods

Reagents

All chemicals unless otherwise indicated were from Sigma-Aldrich Inc. (St. Louis, MO, USA). Cortactin antibodies C001 and 2719 were prepared from rabbit as previously described (Zhan *et al.*, 1994, 1993b). Monoclonal antibody against the myc epitope 9E-10 was a gift from Tom Maciag (Maine Medical Center, Portland, ME, USA).

Cell culture

Embryonic fibroblasts derived from a Src knockout mouse (Src^{-}, clone 1) and embryonic fibroblasts from a normal mouse (Src^{+}, clone 8) were obtained as gifts from Soriano Phillipo (Washington University, Seattle, WA, USA). Cells are cultured in Dulbecco's modified Eagle's medium (DMEM) (Fisher Scientific, Pittsburgh, PA, USA) supplemented with 10% fetal calf serum and antibiotics. Quiescent

confluence in a serum free defined medium supplemented with 10 μg/ml insulin (DMI) for 48 h (Zhan *et al.*, 1986). The stimulation of FGF-1 was initiated by adding recombinant human FGF-1 (10 ng/ml) and heparin (10 units/ml) to the culture.

Cell migration

Quiescent $Src^{-/-}$ and $Src^{+/+}$ cells were stimulated with FGF-1 as described above. After 20 h of stimulation, monolayers of the cells were wounded with a rubber policeman (2 mm wide). The cultures were photographed after 6 h of wounding.

Cell growth assay

Cells were seeded at 1×10^4 cells per well in a 24-well plate containing DMEM supplemented with 10% calf serum. At 8 h after seeding, the medium was changed to DMI supplemented with FGF-1 (10 ng/ml) and heparin (10 μg/ml). In the control samples, DMI without FGF-1 was applied. The treated cells were harvested by trypsinization at the times indicated and counted under a phase-contrast microscope using a hemocytometer. For each time point, three wells of culture were analysed.

Analysis of tyrosine phosphorylation of cortactin

Quiescent cells were stimulated with FGF-1 as described above. At the times indicated, cells were harvested and lysed in RIPA buffer (10 mM Tris-HCl, pH 7.5, 1.0 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1.0 mM sodium orthovanadate, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1.0 mM phenylmethylsulfonyl fluoride), and clarified by centrifugation in a microcentrifuge for 10 min. The supernatants were immunoprecipitated with anti-cortactin antibody 2719 (Zhan *et al.*, 1993b). The immunoprecipitates were then resolved in a SDS-polyacrylamide gel electrophoresis (PAGE) (7.5%, w/v), transferred to a nitrocellouse membrane and further probed with anti-phosphotyrosine antibody (4G10, Upstate Biotechnology Inc. Lake Placid, NY, USA). The blot membrane was stripped and re-probed with the monoclonal cortactin antibody 4F11 (Upstate Biotechnology Inc. Lake Placid, NY, USA) (Wu *et al.*, 1993).

Analysis of cortactin mutants in NIH3T3 cells

The plasmid pMyc-cortactin (encoding a murine cortactin tagged by the *myc* epitope at its NH_2-terminus) and p-Myc-$Cort_{F421F466F482}$ (encoding a murine cortactin where tyrosine residues 421, 466 and 482 were changed to phenylalanine) were previously described (Huang *et al.*, 1998). The two plasmids were transfected into NIH3T3 cells with the Transfection MBS Mammalian Transfection Kit according to the manufacturer's recommended procedure (Stratagene, La Jolla, CA, USA). Stable transfectants were subsequently selected in DMEM containing 10% calf serum and 0.8 mg/ml G418 (Life Technologies, Gaithersburg, MD, USA). Quiescent transfected cells were stimulated with FGF-1 for 12 h and the cell lysates were immunoprecipitated with 9E-10 antibody against the *myc*-epitope. The precipitates were then subjected to immunoblot with a polyclonal phosphotyrosine antibody (Zhan *et al.*, 1993a). The blot was stripped and then re-probed with cortactin antibody C001.

Immunofluorescent analysis

$Src^{-/-}$ and $Src^{+/+}$ cells were grown on fibronectin-coated glass cover slips and maintained in DMI for 48 h. Cells were then stimulated with FGF-1 for overnight, double stained with cortactin antibody C001 and fluorescein-isothiocyanate-phalloidin, and examined by confocal laser microscopy as previously described (Huang *et al.*, 1998).

Abbreviations

F-actin, filamentous actin; FGF, fibroblast growth factor; DMI, defined serum-free medium supplemented with insulin; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein-isothiocyanate; PLC, phospholipase C.

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